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Carbohydrate induced modulation of cell membrane I. Interaction of sialic acid with peripheral blood lymphocytes: a spin label study

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Abstract Sialic acid is now known to serve as ligand for lymphocyte lectins known as selectins. Although its role as a ligand for adhesion molecules has been studied extensively, no studies have been performed to determine the physiological changes in lymphocytes after binding of sialic acid to lymphocyte lectin. We report for the first time that interaction of lymphocytes with sialic acid severely restricts the rotational mobility of the cell surface proteins as well as membrane lipids, as studied by EPR spectroscopy using spin probes. Binding of mucin totally immobilizes the lymphocyte membrane. Surprisingly the binding of sialic acid or mucin also immobilized the aqueous probe TEMPO, indicating an appreciable increase in cytoplasmic viscosity.

Key words: Selectin; Sialic acid interaction; Membrane fluidity; EPR

1. Introduction

It has long been speculated that specific types of sugar chains found on mammalian cell surface must be involved in specific cell–cell interactions [1]. Sialic acids are usually the outermost sugars on mammalian cell surfaces [2]. Several recently discovered adhesion proteins specifically recognize and bind to sugar chains with sialic acids as critical component [3]. One of the family of adhesion molecules consists of selectins, which are involved in leukocyte migration into lymphoid tissues and areas of inflammation [4]. Each selectin has an N-terminal C-type lectin domain that binds specific carbohydrate ligands in a Ca^{2+}-dependent manner. Of the three selectins one is found on almost all types of leukocytes (L selectin), another one is expressed on endothelial cells and platelets (P selectin) and the third is endothelial cell specific (E selectin) [5]. Although numerous reports have appeared delineating the role of selectin in lymphocyte homing, the physiological changes in the lymphocyte after binding of the selectin ligands have never been studied.

We have earlier detected the presence of a sialic acid-binding lectin on peripheral blood lymphocytes [6]. Presuming that binding of sialic acid to this lymphocyte lectin might change lymphocyte physiology we studied the biophysical changes occurring at the lymphocyte membrane by EPR spectroscopy. This report for the first time presents the sialic acid-induced modulation of lymphocyte membrane as studied by EPR spectroscopy.

2. Materials and methods

2.1. Reagents

Histopaque 1077, sialic acid, and all the spin labels, i.e. 5 doxyl stearic acid, 16 doxyl stearic acid, TEMPO and 3 (2 malimidoethyl carbomyl proxyl) were purchased from Sigma Chemical Co., MO, USA. The glass capillaries used were from TOP Syringe Manufacturing Co., Bombay, India. All the other reagents were of analytical grade.

2.2. Methods

Goat peripheral blood lymphocytes were isolated using the Histopaque 1077 density gradient method as described earlier [6]. The cell suspension was adjusted to 1×10^6 cells/ml in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS) with 10 mM $CaCl_2$. From the cell suspension prepared as above, aliquots of 350 μl were made in different tubes. To these tubes was added either sialic acid (40 mM final conc.), mucin (0.0125 mg/ml) or PBS. For saturation studies, sialic acid was varied from 1 to 300 mM final concentration. The cells were incubated with their respective sugar solution for a period of 30 min. After this 50 μl (2×10^{-3} M final conc.) of spin label (which included 5DS, 16DS, MECP and TEMPO) were added. The suspension was incubated at 37°C for 30 min. Immediately after the lapse of the incubation, 50 μl $NiCl_2$ (final conc. 500 mM) was added to each sample and incubation was continued for another 10 min. The cells were washed three times with PBS to remove excess label. The pellet was resuspended in 100 μl of PBS. From these suspensions approximately 50 μl was transferred to glass capillaries and one end sealed with plasticine, taking care not to trap air bubbles in it.

2.3. EPR spectroscopy

EPR spectra were recorded on a Varian E-104, EPR spectrometer equipped with a TM_{110} cavity. Instrument settings employed were as follows: scan range 100 G; field set 3237G; temp. 27°C; time constant 1 s; scan time 1 s; modulation amplitude 2 G for 16DS and TEMPO and 1 G for 5DS and MECP; modulation frequency 100 kHz; microwave power 5 mW; microwave frequency 9.01 GHz; receiver gain 2.5×10^4. The rotational correlation time T_c, expressed in seconds was calculated by employing the equation $T_c = k \cdot w_o (h_o/h_{-1})^{1/2} - 1$ (where w_o is the mid field line width, h_o and h_{-1} are the mid and high field line heights, respectively, and $k = 6.51 \times 10^{-10}$ and is a proportionality constant. Isotropic nitrogen hyperfine coupling constant, a_N, expressed in Gauss is measured by calculating the distances between the low field absorption line and the mid field absorption line at cross-over. The order parameter (S_3), is obtained from the anisotropic hyperfine splitting $T_{\parallel}$ and $T_{\perp}$ by using the equation $S = T_{\parallel} - T_{\perp}/T_{\parallel} + T_{\perp}$. The lateral diffusion coefficient (W_{ex}) was measured by taking the width w_o of the mid field line in Gauss. W_{ex} is directly proportional to w_o [10].

The experiments were repeated four to six times and data were subject to statistical analysis using the STAT-P GW-BASIC-Rev 1.02 programme.

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Abbreviations: 5DS, 5 doxyl stearic acid; 16DS, 16 doxyl stearic acid; MECP, 3 (2 malimidoethyl carbomyl Proxyl); TEMPO, 2,2,6,6 tetra methyl piperidinooxyl; PBS, 0.01 M phosphate buffer, pH 7.4, with 0.15 M NaCl.

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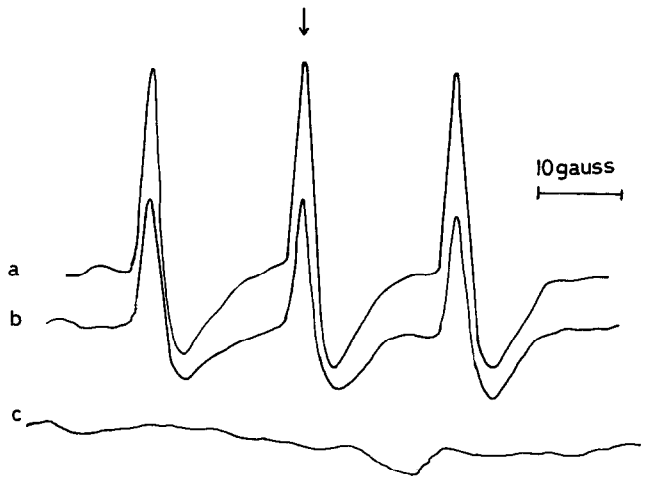

Fig. 1. EPR spectra of goat peripheral blood lymphocytes labeled with the spin label MECP. Instrument settings were as described in section 2. (a) Lymphocytes in PBS. (b) Lymphocytes in sialic acid (40 mM final conc.). (c) Lymphocytes in mucin (0.0125 mg/ml).

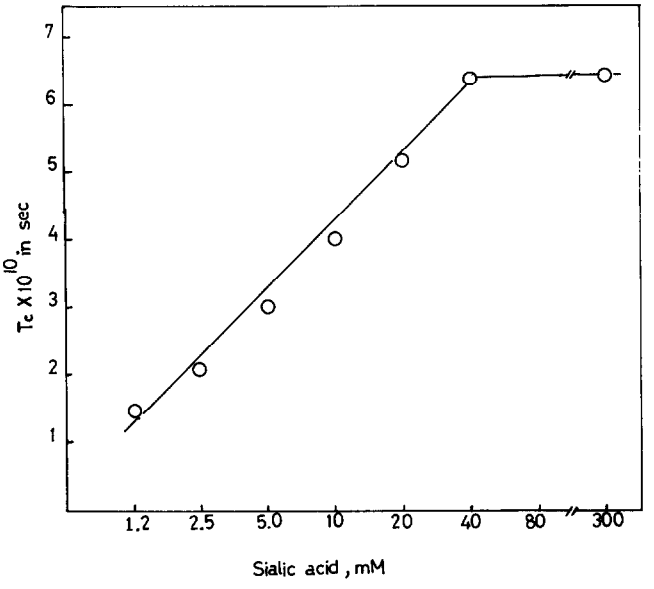

Fig. 2. Plot of the increase in T_c values with increasing sialic acid concentration. The spin label was 16DS.

3. Results and discussion

The changes in lymphocyte membrane fluidity associated with binding of sialic acid (or its derivative like mucin) to cell surface lectins were studied by employing a variety of spin-labelled compounds to probe various environments of the lymphocytes. The stearic acid spin labels 5DS and 16DS were used as probes for the hydrocarbon portion of the membrane bilayer. TEMPO was used as a probe for the aqueous compartments, while MECP was used to probe surface proteins with the reactive NH_2 group. EPR spectra obtained were analysed by computing the spectral parameters, which included nitrogen hyperfine coupling constant (a_N), rotational correlation time (T_c), order parameter (S_3), and an empirical measure of the lateral diffusion coefficient (W_{ex}).

The results obtained are presented in Figs. 1–5. It appears that the binding of sialic acid to membrane lectins present on lymphocytes results in a decrease in fluidity of the lymphocyte membrane, measured in terms of the rotational correlation time T_c. T_c is the measure of the degree of immobilization of the spin label, hence a measure of the local viscosity. The reciprocal of T_c ($1/T_c$) will thus denote the local 'fluidity'; a higher $1/T_c$ value denotes a higher fluidity. The spin labels used in this study were selected so as to study the mobility changes in cell membrane proteins (the protein domain), the lipid bilayer (the lipid domain), and the aqueous compartment (the cytoplasm). All the spin labels used indicated that binding of sialic acid to lymphocytes results in a decrease in the degree of mobilization of the spin label. Fig. 1a,b shows the EPR spectra for MECP incorporated in lymphocytes. On binding of sialic acid the $1/T_c$ value decreases from $1.68 \pm 0.16 \times 10^{10}$ (in PBS) to 1.28 ± 0.10^{10} (see Table 1). We see a considerable reduction and broadening of lines in the presence of sialic acid. Further, if mucin is used in place of sialic acid the spectra become almost flat, indicating total immobilization of the label (Fig. 1c). Mucin is a polymer containing repeating units of sialic acid [7]. It appears that multiple binding sites with mucin cross-links the membrane lectins, thereby totally reducing the mobility. Surprisingly the S_3 value (order parameter, reflecting the molecular ordering or 'packing' of the micro environment) does not change significantly, indicating that, although rotational mobility of the protein molecules is considerably hindered after binding of sialic

Table 1
Changes in $1/T_c$ values after treatment of lymphocytes with sialic acid

Spin probe used	Lymphocytes in PBS ($1/T_c \times 10^{-10}$)	Lymphocytes in sialic acid ($1/T_c \times 10^{-10}$)	Level of significance (P)
MECP	1.68 ± 0.16	1.28 ± 0.10	<0.001
5DS	0.52 ± 0.003	0.41 ± 0.002	<0.001
16DS	1.34 ± 0.319	0.16 ± 0.001	<0.001
TEMPO	0.93 ± 0.16	0.68 ± 0.005	<0.05

All experiments were independently repeated 6 times: the results are highly significant. Calculations for $1/T_c$ could not be done for mucin as the spectra were totally flat.

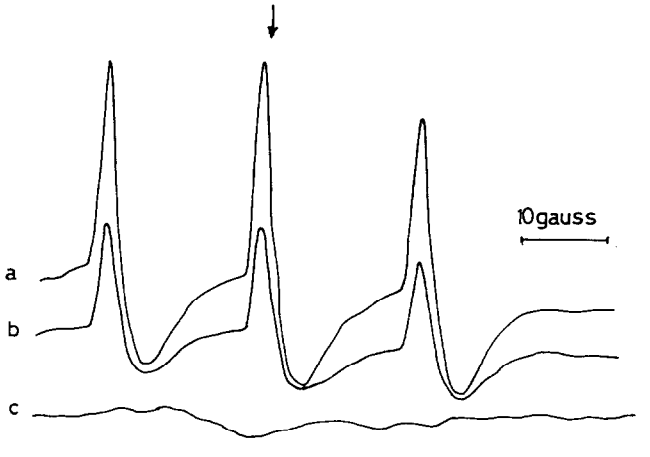

Fig. 3. EPR spectra of goat peripheral blood lymphocytes labeled with the spin label 5DS. Instrument settings were as described in section 2. (a) Lymphocytes in PBS. (b) Lymphocytes in sialic acid (40 mM final conc.). (c) Lymphocytes in mucin (0.0125 mg/ml).

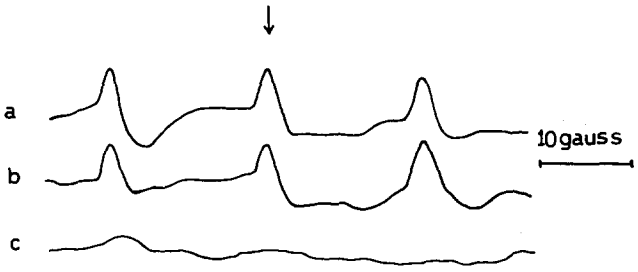

Fig. 4. EPR spectra of goat peripheral blood lymphocytes labeled with the spin label 16DS. (a) Lymphocytes in PBS. (b) Lymphocytes in sialic acid (40 mM final conc.). (c) Lymphocytes in mucin (0.0125 mg/ml).

acid or mucin, there is no major change in the packing of molecules in the existing crystalline conformation. Generally the selectins are members of a family of animal lectins, known as C-type lectins, which require Ca^{2+} ions for their activity [8]. To test if this change in $1/T_c$ is due to Ca^{2+}-sensitive binding of sialic acid to lectins, the lymphocytes were initially dispersed in PBS containing 10 mM EDTA instead of 10 mM $CaCl_2$: the rest of the procedure was followed as usual. We found that if EDTA was present in place of $CaCl_2$ no change in $1/T_c$ values was observed when the cells were incubated with sialic acid (data not shown). This clearly indicates that a calcium-sensitive C-type lectin is binding to sialic acid and producing the observed effects.

To further analyse if this is a specific receptor–ligand interaction we studied the dose-dependent changes in the T_c value with respect to the addition of sialic acid in increasing concentrations from 1 to 300 mM (see Fig. 2). The spin label used was 16DS. We find that the T_c value increases linearly with addition of increasing concentrations of sialic acid, with saturation being achieved around 40 mM (for 1×10^6 cells/ml). A further 100-fold increase in sialic acid concentration produced no further increase in T_c values. The change in $1/T_c$ values was the same even if the spin label were incorporated into the cells before incubation with sialic acid. Incubation of free sialic acid or mucin with free spin label produced no change in their EPR spectra.

Apart from labelling membrane proteins the lipid bilayer was also probed using 5DS and 16DS (see Figs. 3 and 4). A similar decrease in $1/T_c$ values was obtained with 16DS. Among all the spin labels used an 8-times decrease in $1/T_c$ values was obtained with 16DS, i.e. the value changed from $1.34 \pm 0.319 \times 10^{10}$ in PBS to $0.16 \pm 0.001 \times 10^{10}$ in sialic acid. Here also the S_3 values

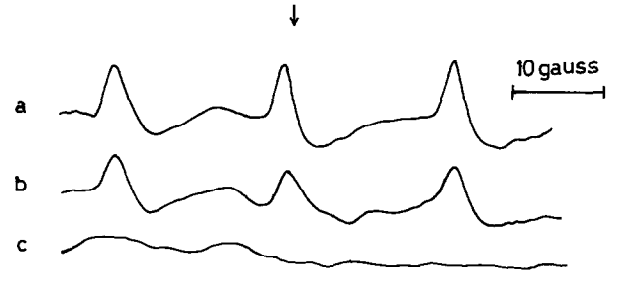

Fig. 5. EPR spectra of goat peripheral blood lymphocytes labeled with the spin label TEMPO. (a) Lymphocytes in PBS. (b) Lymphocytes in sialic acid (40 mM final conc.). (c) Lymphocytes in mucin (0.0125 mg/ml).

did not change significantly. The most interesting results were obtained with the aqueous label TEMPO. TEMPO is a water-soluble label which can pass through the membrane to the cytoplasm, and thus the signals generated by TEMPO denotes the fluidity status of the cytoplasm [9]. Sialic acid changed the $1/T_c$ value of TEMPO from $0.93 \pm 0.16 \times 10^{10}$ to $0.68 \pm 0.005 \times 10^{10}$, thus indicating a decrease in fluidity. Mucin again totally immobilized the label (see Fig. 5). TEMPO is known to be catabolized by living cells [9]. To ensure that loss of TEMPO signals is not due to its degradation but rather to immobilization, the TEMPO-labelled, mucin-treated cells were sonicated to disrupt the cell membrane so as to release the cytoplasm into the extracellular buffer, thereby increasing fluidity. TEMPO signals reappeared on sonication (data not shown). This clearly indicates that the TEMPO was not catabolized but rather totally immobilized, thus producing a flat spectra.

Our results clearly indicate that binding of sialic acid or mucin to peripheral blood lymphocytes markedly reduces the rotational mobility of membrane proteins as well a lipids. The immobilization produced by mucin was expected, as mucin has a large number of sialic acid groups which will cross-link all the sialic acid-binding proteins in the cell membrane, resulting in restricted mobility. The decrease in fluidity on binding of (monomeric) sialic acid could be due two reasons, one being a confirmational change produced in the sialic acid-binding protein leading to decreased rotational freedom, and the second being the possibility of the cross-linking of more than one sialic acid-binding protein with one sialic acid molecule. Further studies are required to delineate the exact process. We also feel that this binding is also producing a signal changing lymphocyte physiology, which is expressed in decreased mobility of TEMPO, indicating a rise in cytoplasmic viscosity. This observation also requires further exploration. Our unpublished results indicate that sialic acid produces similar changes in other immune cells, like mice and hamster splenic lymphocytes, as well as in non-immune cells like spermatozoa. We also feel that this technique has great potential for the study of various parameters, such as association constants in lectin–carbohydrate interactions.

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